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(21) International Application Number: PCT/CA98/00749 (22) International Filing Date: 5 August 1998 (05.08.98) (30) Priority Data: 2,212,315 5 August 1997 (05.08.97) CA (71) Applicant (for all designated States except US): MCGILL UNIVERSITY [CA/CA]; 845 Sherbrooke Street West, Montreal, Quebec H3A 1B1 (CA). (72) Inventors; and (75) Inventors/Applicants (for US only): SARAGОВI, H., Uri [US/CA]; 342 Lansdowne Avenue, Westmount, Quebec H3Z 2L4 (CA). MALIARTCHOUK, Sergei [CA/CA]; Apartment 405, 5700 Cavendish, Cote Saint-Luc, Quebec H4W 1S8 (CA). (74) Agents: COTE, France et al.; Swabey Ogilvy Renault, Suite 1600, 1981 McGill College Avenue, Montreal, Quebec H3A 2Y3 (CA).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: PHARMACEUTICAL COMPOSITION WITH NEUROTROPHIC-LIKE BIOLOGICAL ACTIVITY		
(57) Abstract		
<p>The present invention relates to a pharmaceutical composition having a neurotrophic-like biological activity which comprises a first compound which binds to and/or activates at least one trk receptor, and a second component which binds to and/or activates p75 receptor, in association with a pharmaceutically acceptable carrier. The neurotrophic-like biological activity may be that of NGF. The trk receptor may be selected from the group consisting of trkA, trkB and trkC.</p>		

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**PHARMACEUTICAL COMPOSITION WITH
NEUROTROPHIC-LIKE BIOLOGICAL ACTIVITY**

BACKGROUND OF THE INVENTION

5 (a) Field of the Invention

The invention relates to a pharmaceutical composition having a neurotrophic-like biological activity which comprises a first compound which binds to and/or activates at least one trk receptor, and a second component which binds to and/or activates p75 receptor.

10 (b) Description of Prior Art

Nerve Growth Factor (NGF) is a 26 kDa dimeric polypeptide which binds two receptors characterized on the basis of their binding affinity. One NGF receptor is a 140 kDa protein (p140 TrkA) with intrinsic tyrosine kinase enzymatic activity. NGF binds TrkA with intermediate affinity (K_d 10^{-10} to 10^{-11} M). Another receptor is a 75 kDa protein (p75) that is bound by NGF and other neurotrophins such as BDNF with lower affinity (K_d $\sim 10^{-9}$ M).

Co-expression of TrkA and p75 on the cell surface leads to the formation of a limited number of high affinity NGF binding sites (K_d $\sim 10^{-12}$ M), that are presumably composed of p75-TrkA heteromers. However, biochemical detection of p75 and TrkA heteromers has not been conclusive.

While expression of TrkA alone is sufficient for cellular responses (Nebreda AR et al. (1991) Science 252:558-563; Rovelli G et al. (1993) Proc. Nat. Acad. Sci. (USA) 90:8717-21), p75 can regulate TrkA-ligand interactions and signal transduction (Hempstead BL et al. (1989) Science 243:373-375; Verdi JM et al. (1994) Neuron 12:33-745; Dobrowsky RT et al. (1995) J. Biol. Chem. 270:22135-42). Moreover, p75 activates its own signaling pathway (Carter BD et al. (1996) Science 272:542-545; Canossa M et al. (1996) EMBO J. 15:3369-

3376; Cortazzo, MH et al. (1996) J. Neurosci 16: 3895-3899). It has been suggested that in certain systems ligand-bound p75 receptors may activate apoptotic signals, while in other systems it is unbound p75 receptors that activate apoptosis.

One problem in elucidating the molecular structure of the functional NGF receptor, in determining the individual role of each receptor, and a putative cross-modulation between TrkA and p75 has been the difficulty in obtaining high affinity ligands that completely discriminate between the receptors. Mutant neurotrophins that bind Trk receptors preferentially over p75 function like wild type neurotrophins in biological assays. However, NGF appears to dock onto multiple sites of TrkA, [the IgG-like domain and/or the leucine zipper domain]. Ligand binding to multiple TrkA sites may cause signaling and may lead to p75 immobilization and p75-independent signals.

The Applicants have previously described a monoclonal antibody (mAb) 5C3 that binds a restricted epitope of TrkA with high affinity, and acts as a full agonist (when compared with NGF) on cells that express TrkA but do not express p75 (LeSauter L et al. (1996) J Neurosci 16:1308-1316). In the present study, combinations of the TrkA-specific mAb 5C3 and the p75-specific mAb MC192 (Chandler CE et al. (1984) J. Biol. Chem. 259:6882-6889) were used as ligands to analyze NGF receptor function in functional and biochemical assays. These mAbs maintain high binding affinity irrespective of and unaffected by expression of co-receptors.

It would be highly desirable to be provided with a pharmaceutical composition having a neurotrophic-like biological activity.

SUMMARY OF THE INVENTION

One aim of the present invention is to provide a pharmaceutical composition having a neurotrophic-like biological activity.

5 Surprisingly, and in accordance with the present invention, the data supports the hypothesis that NGF-trophic signals are mediated by TrkA, and that unbound p75 modulates TrkA trophic function. More importantly, the data shows that optimal agonistic ligand mimicry
10 for a multireceptor complex can be achieved by a combination of the natural ligand and an anti-receptor antibody, or by a combination of two antibodies against different receptors. This information will be useful in the design of artificial agonists in multireceptor
15 systems, including neurotrophin receptors.

In accordance with the present invention there is provided a pharmaceutical composition having a neurotrophic-like biological activity which comprises a first compound which binds to and/or activates at least
20 one trk receptor, and a second component which binds to and/or activates p75 receptor, in association with a pharmaceutically acceptable carrier.

The neurotrophic-like biological activity may be that of NGF, NT-3, neurotrophins such as BDNF, CNTF,
25 GDNF, and cytokines.

The trk receptor may be selected from the group consisting of trkA, trkB and trkC.

BRIEF DESCRIPTION OF THE DRAWINGS

30 Figs. 1A and 1B illustrate changes in apoptotic DNA degradation;

Figs. 2A-2E illustrate optimal TrkA tyrosine phosphorylation by concomitant binding of p75 and TrkA;

Fig. 3 illustrates that TrkA binding peptides
35 protect cell from death and that combination of p75

ligands and TrkA binding peptides synergizes and affords nearly 100% protection;

Figs. 4A and 4B illustrate NMR spectroscopy of C(92-96) peptide mimetic; and

5 Figs. 5A and 5B illustrate that NGF-like signal can be promoted by small molecules and antibodies ligands of TrkA and p75.

DETAILED DESCRIPTION OF THE INVENTION

10 Nerve Growth Factor (NGF) receptor-mediated signaling was studied using specific monoclonal antibodies (mAbs) as ligands that discriminate between the receptors TrkA and p75. MAb-induced trophic signals were compared with the signals of the natural ligand
15 NGF. In cells expressing TrkA but no p75 receptors (TrkA⁺ p75⁻), binding of TrkA with mAb 5C3 leads to optimal signals. In cells expressing both TrkA and p75 (TrkA⁺ p75⁺), binding of TrkA with mAb 5C3 leads to significant but suboptimal signals; and optimal trophic
20 signals are obtained by concomitant binding of TrkA and p75 with mAbs 5C3 and MC192. In TrkA⁺ p75⁺ cells binding of anti-p75 mAb MC192 also enhances the trophic effect of suboptimal concentrations of NGF. In contrast, in cells expressing p75 receptors singly (TrkA⁻
25 p75⁺), binding with mAb MC192 or NGF causes very limited or no trophic effects. Thus, the data supports the hypothesis that unbound p75 may modulate TrkA trophic signals. Importantly, the data also demonstrates for the first time that in multireceptor systems appropriate combinations of anti-receptor mAbs can fully mimic
30 the signals of a polypeptide growth factor.

Cell cultures

35 Rat PC12 pheochromocytomas cells express p75 and TrkA, B104 rat neuroblastoma cells express ~50,000 surface p75 receptors/cell and none of the Trks (TrkA⁻

p75⁺); 4-3.6 cells are B104 cells transfected with human trkA cDNA and express equal levels of surface p75 and TrkA (TrkA⁺ p75⁺). The C10 cell line is a selected subclone of 4-3.6 expressing ~50,000 surface TrkA
5 receptors but no detectable surface p75 (TrkA⁺ p75⁻). Lack of detectable surface p75 receptors on C10 clones was assessed by FACScan analysis (with a sensitivity of <500 receptors/cell). All cell lines were maintained in
10 RPMI media (GIBCO, Toronto, Ontario) supplemented with 5% fetal bovine serum (FBS) and antibiotics. Appropriate drug selection was added to 4-3.6 and C10 cells.

Antibodies as NGF receptor ligands

Anti-rat p75 mAb MC192 (IgG1; Chandler CE et al. (1984) J. Biol. Chem. 259:6882-6889) and anti-human
15 TrkA mAb 5C3 (IgG1, LeSauter L et al. (1996) J Neurosci 16:1308-1316) ascites were purified with Protein G Sepharose (Pharmacia, Baie d'Urfe, Quebec), dialyzed against phosphate buffered saline and stored at -20°C. MAb 5C3 is agonistic and can fully substitute for NGF
20 in E25 cells expressing TrkA but not p75 (LeSauter L et al. (1996) J Neurosci 16:1308-1316). Further characterization of mAb 5C3 is published in LeSauter L et al. article ((1996) J Neurosci 16:1308-1316). Purified mAbs were characterized by SDS-PAGE under non-reducing
25 or reducing (100 mM 2-mercaptoethanol) conditions to >95% purity.

Binding assays with directly labeled mAbs 5C3 and MC192 demonstrated that each antibody binds to its receptor with relative affinity and saturation profiles
30 irrespective of whether the other receptor is expressed and bound. For example, mAb 5C3 binds similarly to TrkA⁺ p75⁻ cells or TrkA⁺ p75⁺ cells regardless of whether mAb MC192 is present. This is not unusual or unexpected.

Protection from cell death

5,000 cells/well in protein-free media (PFHM-II, GIBCO, Toronto, Ontario) containing 0.1% BSA (Crystalline fraction V, Sigma, St. Louis MO) were added to 96 well plates (Falcon, Mississauga, Ontario). The cultures were untreated or supplemented with serial dilutions of neurotrophins (positive control), test mAbs, or mouse IgG (negative control). The survival profile of the cells was quantitated using the MTT colorimetric assay after 48-72 hours. Percent protection was standardized relative to 1 nM NGF concentrations using the MTT optical density (OD 590 nm) and following formula:

$$[(OD_{\text{test}} - OD_{\text{untreated}}) / (OD_{1 \text{ nM NGF}} - OD_{\text{untreated}})] \times 100.$$

The OD of untreated samples (SFM only) were ~10% of 1 nM NGF control.

Some survival experiments were also carried out in the presence of various concentrations of the tyrosine kinase inhibitor K252a. The concentrations of K252a used were previously reported.

DNA fragmentation and apoptosis

Apoptotic death was confirmed by analysis of DNA fragmentation patterns by extraction of genomic DNA. Equal amounts of DNA for each condition were resolved in a 1.5% agarose gel and visualized with ethidium bromide. Note that DNA isolated from apoptotic PC12 cells often does not appear as a typical apoptotic ladder.

Tyrosine phosphorylation assays

The tyrosine phosphorylation of TrkA was assayed after a 15 min. treatment of 4-3.6 cells with the indicated agent(s). Analysis was done by Western Blot of whole cell lysates with the enhanced chemoluminescence detection system (ECL, Amersham, Oakville, Ontario) as described (LeSauter L et al. (1996) J Neurosci 16:1308-1316) using anti-phosphotyrosine mAb 4G10 (UBI,

NY); or affinity purified polyclonal antisera DF-49 recognizing phosphotyrosine PY490 of TrkA which forms the Shc recognition/docking site on TrkA. Quantitation of protein loading was done with the Bio-Rad Detergent
 5 Compatible Protein Assay reagent (Bio-Rad Laboratories), and by coomassie blue staining of gels. Bands in X-ray films were quantified by densitometry [Scanmaster3+ scanner (Howtec Inc., NH) and MSCAN software (Scanalytic, CSP Inc., Hudson, NH)]. Band intensi-
 10 ties were standardized using the relative optical density of NGF treatment in each film as 100%. Statistical analysis of densitometry of 3-5 gels were done by paired student t-tests.

Functional Consequences of NGF Receptor Binding

15 Cells undergo apoptotic death when cultured in serum free media (SFM) (Table 1).

Table 1

p75 binding does not protect from apoptotic death in SFM

20

	TREATMENT IN SFM CULTURES	PC12 (TrkA ⁺ p75 ⁺)	B104 (TrkA ⁻ p75 ⁺)	4-3.6 (TrkA ⁺ p75 ⁺)
1	mouse IgG	0 ± 2.3	0.5 ± 0.7	0 ± 2.2
2	1 nM NGF	100 ± 5.1	-3 ± 2.2	100 ± 4.6
3	100 pM NGF	80 ± 5.5	1 ± 1.9	87.4 ± 4.5
4	10 pM NGF	40 ± 3.4	2 ± 1.7	52.3 ± 4.7
5	1 pM NGF	12 ± 1.1	-0.5 ± 1.2	10.1 ± 5.5
6	2 nM BDNF	not tested	1 ± 1.3	2.1 ± 1.7
7	200 pM BDNF	not tested	2.5 ± 1.3	0.9 ± 1.2
8	20 pM BDNF	not tested	1.6 ± 1.3	3.4 ± 2.2
9	2 pM BDNF	not tested	0 ± 0.7	0.5 ± 3.7
10	MC192 10 nM	2 ± 1.2	0.7 ± 0.8	1.2 ± 2.3
11	MC192 1 nM	-1 ± 3.4	0.6 ± 2.3	3.7 ± 3.1
12	5 % serum	157 ± 0.9	100 ± 7.7	148 ± 7.2

PC12, B104, and 4-3.6 cells were cultured in serum free media (SFM), supplemented with test or control ligands as indicated. Cell protection was quantitated after 48 hours by measuring optical density using the MTT colorimetric assay. Data was standardized relative to optimal NGF treatment (PC12 and 4-3.6 cells). B104 cells do not respond to NGF, thus in this assay they were standardized with respect to 5% serum. A representative experiment is shown (average \pm standard deviation, n=4) from >3 independent experiments.

B104 cells expressing p75 but not TrkA were not protected by p75 ligands [neurotrophins NGF and BDNF (rows 2-9), or by various concentrations of anti-p75 mAb MC192 (rows 10,11)]. Lack of significant p75 ligand-induced protection in SFM was irrespective of TrkA expression, and apoptotic death occurred in p75⁺ TrkA⁺ PC12 cells (Table 1, rows 10,11); and in p75⁺ TrkA⁺ 4-3.6 cells (Table 1, rows 6-11). In contrast, NGF binding to TrkA protected cells from apoptotic death in SFM (Table 1, rows 2-5). NGF-mediated protection of PC12 and 4-3.6 cells was dose dependent and consistently suboptimal at ~1-10 pM (Table 1, rows 4 and 5). Standard cell culture conditions containing 5% serum (Table 1, row 12) afford both proliferation and survival. Therefore, higher readings are detected when compared with 1 nM NGF, which in SFM preferentially acts as a survival factor.

Next, cells expressing p75 and human or rat TrkA receptors were used to test potential synergy of mAb MC192 as a p75 ligand and suboptimal NGF doses (5 pM) as a preferential high affinity ligand. MAb MC192 alone affords very limited (or insignificant) protection in SFM (Table 2, rows 4-6; also see Table 1); and 5 pM NGF alone affords suboptimal cell protection ranging from ~30-50% (Table 2, row 3; also see Table 1).

Table 2

Concomitant p75 and TrkA binding protects cells
from apoptotic death

5

	TREATMENT ADDED TO SFM CULTURES	PC12 (TrkA ⁺ p75 ⁺)	4-3.6 (TrkA ⁺ p75 ⁺)
1	mouse IgG	0 ± 1	0 ± 1.5
2	1 nM NGF	100 ± 6.5	100 ± 4.8
3	5 pM NGF	28 ± 8.4	48 ± 3.5
4	MC192 10 nM	2 ± 1.5	1 ± 4.1
5	MC192 1 nM	6 ± 2.7 ^a	6 ± 3.5 ^a
6	MC192 0.1 nM	1 ± 2.0	1 ± 4.2
7	5 pM NGF + MC192 10 nM	49 ± 3.3	85 ± 6.5
8	5 pM NGF + MC192 1.0 nM	86 ± 7.4	108 ± 5.6
9	5 pM NGF + MC192 0.1 nM	26 ± 5.1	55 ± 4.5

^a the small increase in survival induced by mAb MC192 is statistically significant.

Assays were done as described in Table 1 legend. MAb MC192 synergizes with suboptimal (5 pM) NGF in protecting PC12 and 4-3.6 cells from apoptotic death in SFM (rows 7 and 8).

5 pM NGF + mAb MC192 synergized to significantly increase cell protection in SFM (Table 2, rows 7-9). This protection was dependent on the concentration of mAb MC192, and was maximal at 0.2 mg/ml (1 nM) (Table 2, row 8). MAb MC192 concentrations ranging from 0.1 nM to 1 mM were tested but only some concentrations are shown for clarity. At 2 mg/ml (10 nM) or higher concentrations mAb MC192 afforded limited synergy (Table 2, row 7); and at 0.02 mg/ml (0.1 nM) or lower concentrations it did not synergize with NGF (Table 2, row 9). Thus, a bell-shaped dose response resulted wherein low or high concentrations of mAb do not afford synergy with 5 pM NGF.

Similar tests were performed with 4-3.6 cells (human TrkA⁺ p75⁺) and C10 cells (a sorted subclone of

4-3.6 cells, that express human TrkA but is p75⁻). 4-3.6 and C10 clones express similar number of surface human TrkA receptors. In these cells it is possible to replace NGF with mAb 5C3 as a test ligand for human TrkA (Table 3).

Table 3

Concomitant ligand binding of p75 and TrkA synergizes in trophic signals

10

	TREATMENT ADDED TO SFM CULTURES	4-3.6 (TrkA ⁺ p75 ⁺)	C10 (TrkA ⁺ p75 ⁻)
1	mouse IgG	0 ± 3.4	0 ± 1.7
2	1 nM NGF	100 ± 5.3	100 ± 3.7
3	100 pM NGF	89 ± 6.6	35 ± 3.1
4	10 pM NGF	52 ± 3.6	7 ± 3.1
5	1 pM NGF	4 ± 4.4	0 ± 2.4
6	5 nM MC192	16 ± 5.7 ^a	1 ± 1.2
7	0.5 nM MC192	8 ± 4.0	0 ± 1.8
8	5 nM 5C3	42 ± 3.0	79 ± 5.2
9	0.5 nM 5C3	20 ± 5.5	64 ± 5.3
10	5 nM 5C3 + 5 nM MC192	78 ± 2.7	73 ± 3.8 ^c
11	5 nM 5C3 + 0.5 nM MC192	118 ± 3.1 ^b	59 ± 4.9 ^c
12	0.5 nM 5C3 + 5 nM MC192	65 ± 6.8	62 ± 1.6 ^c
13	0.5 nM 5C3 + 0.5 nM MC192	96 ± 2.3	64 ± 1.4 ^c

^a the small increase in survival induced by mAb MC192 is statistically significant.

^b the survival higher than 100% is statistically significant from 1 nM NGF.

15 ^c not statistically significant from each other.

Assays were done as described in Table 1 legend. Binding of p75 and TrkA with mAbs MC192 and 5C3 respectively (rows 9-12) synergize in protecting 4-3.6 cells from apoptotic death, while binding of TrkA with mAb 5C3 alone (rows 8 and 9) affords suboptimal protection. In contrast, C10 cells are better protected by binding TrkA with mAb 5C3 alone (rows 8 and 9).

20

Combinations of mAbs 5C3 and MC192 afforded optimal 4-3.6 cell protection (Table 3, row 10-13) which is comparable to that afforded by optimal NGF (Table 3, row 2). Synergy by combination of mAbs 5C3 and MC192 is demonstrated by significantly higher protection than treatment with either mAb alone (Table 3, rows 6-9). Interestingly, while binding of TrkA with mAb 5C3 alone affords only ~20-40% protection to 4-3.6 cells; similar treatment of C10 cells affords 65-80% protection in SFM (Table 3, rows 8 and 9). MAb 5C3 concentrations ranging from 0.01 mg/ml to 5 mg/ml (0.05 nM to 250 nM) were tested but only some concentrations are shown for clarity.

Consistent with C10 cells lacking surface p75, the combination of mAbs MC192 and 5C3 does not enhance the effect of mAb 5C3 alone (Table 3, rows 10-13). Expectedly, C10 cells are less responsive to low doses of NGF than 4-3.6 cells (Table 3, rows 3-5) because they lack detectable p75. Further, no synergy was observed in C10 cells when mAb MC192 and 5 pM NGF were tested in combination.

To assess whether trophic signals leading to cell survival in SFM were mediated via a tyrosine kinase activity, the K252a inhibitor was used (Table 4).

Table 4

K252a inhibits NGF receptor-mediated trophic signals

K252a (nM)	% CELL SURVIVAL IN SFM SUPPLEMENTED WITH		
	NGF	5C3	5C3+192
0	100 ± 9	50 ± 3	112 ± 4
50	60 ± 4	32 ± 3	67 ± 5
500	32 ± 4	13 ± 2	43 ± 2

Assays were done as described in Table 1 legend. 4-3.6 cell survival in SFM achieved by incubation with the indicated ligands. Optimal ligand concentrations were used as per Table 3 (1 nM NGF, 5 nM 5C3 mAb and 5 nM 5C3 + 0.5 nM MC192 mAbs). Cells were challenged with various concentrations of K252a and % survival was calculated using 1 nM NGF as 100% standard. K252a inhibits both NGF and mAb-mediated survival in a dose dependent manner and to a similar relative degree.

As expected, K252a inhibited trophic survival induced by 1 nM NGF. K252a also inhibited trophic survival induced by optimal concentrations of mAb 5C3 or by optimal combinations of mAbs 5C3 + MC192. Inhibition by K252a was dose dependent. The highest concentration of K252a tested (500 nM) was not toxic to 4-3.6 cells.

Analysis of the degradation pattern of genomic DNA confirmed the apoptotic nature of cell death in SFM for 4-3.6 and PC12 cells (Fig. 1) and for B-104 cells. Genomic DNA was extracted from (Fig. 1; A) 4-3.6 cells or (Fig. 1; B) PC12 cells cultured as indicated for 48 hours in SFM. Equal amounts from each sample were resolved on a 1.5% agarose gel and visualized with ethidium bromide. Standard molecular markers (M) are shown. A typical apoptotic DNA ladder is seen for 4-3.6 cells, but PC12 DNA is more smeared and difficult to isolate as a ladder. Antibody concentrations were selected from optimal survival assays (e.g. Table 3), namely 5 nM mAb 5C3 and 0.5 nM mAb MC192. 5 pM NGF was suboptimal in survival assays and some DNA laddering is expected (panel B); and that DNA laddering is ablated when NFG is combined with 0.5 nM mAb MC192.

The absence or presence of DNA degradation correlated conclusively with protection or lack of protection from death for all treatments and for all cell lines (Tables 1-3).

In 4-3.6 cells, no DNA degradation is seen upon culture with 5% serum or with mAbs 5C3+MC192; while a small amount of DNA degradation is seen for 4-3.6 cells treated with mAb 5C3 (Fig. 1A). In contrast, extensive apoptotic DNA degradation is seen when 4-3.6 cells are cultured with SFM or mAb MC192 alone (Fig. 1A).

In PC12 cells, no DNA degradation is seen upon culture with 5% serum or with 5 pM NGF+10 nM mAb MC192. PC12 cells treated with 5 pM NGF alone do have limited DNA degradation (Fig. 1B), as expected because this concentration of NGF affords suboptimal survival. PC12 cells cultured with SFM or mAb MC192 alone show extensive DNA degradation (Fig. 1B).

TrkA tyrosine phosphorylation

To further analyze the signaling mechanism of the antibody-based ligand combinations, TrkA tyrosine phosphorylation (PY) was studied. This was done by western blot analysis of whole cell lysates with antibodies against phosphotyrosine (a-PY), or with antibodies that bind phosphotyrosinylated TrkA within the Shc recognition/docking site [phosphotyrosine 490 of TrkA (a -PY490, DF-49 antibody)].

Initial experiments were designed to resolve the concentration of mAb 5C3 that affords optimal PY of TrkA (Table 5).

Table 5

TrkA tyrosine phosphorylation in response to MAb 5C3

	Cells	C10 CELLS		4-3.6 CELLS	
		PYtotal	PY490	PYtotal	PY490
1	no ligand	11	1	4	1
2	NGF 1 pM	12	1	7	5
3	NGF 10 pM	12	1	44	33
4	NGF 100 pM	36	45	93	61
5	NGF 1 nM	100	100	100	100
6	5C3 0.05 nM	10	1	5	1
7	5C3 0.5 nM	40	40	32	21
8	5C3 5 nM	91	71	45	43
9	5C3 50 nM	35	49	39	21

5

Cells were untreated (row 1) or treated with the indicated concentrations of NGF (rows 2-5); or mAb 5C3 (rows 6-9); for 15 min at 37°C. Ligand concentrations were selected based on survival assays (e.g. Table 3). Equal amounts of protein from whole cell lysates were resolved by SDS-PAGE and analyzed by western blotting with anti-phosphotyrosine (anti-PY) or with a-PY490 blot (DF-49 sera) recognizing specifically the Shc binding site of TrkA. Band intensities were analyzed by densitometry and standardized using the relative optical density of 1 nM NGF treatment as 100%. Data from a representative western blot is shown.

A 15 minute treatment with mAb 5C3 at 1 mg/ml (5 nM) induced optimal TrkA PY and TrkA PY490 in C10 (TrkA⁺ p75⁻) and 4-3.6 cells (TrkA⁺ p75⁺). This was consistent with previous survival data (e.g. Table 3). However, 5 nM mAb 5C3 was less efficient at phosphorylating TrkA when compared with 1 nM NGF (Table 5, row 5). This result is also consistent with previous survival data.

As expected, TrkA phosphorylation in response to low NGF concentrations (Table 5, rows 2-4) was decreased in C10 cells compared with 4-3.6 cells, because C10 cells do not express p75 receptors. In contrast, TrkA phosphorylation in response to mAb 5C3 was always stronger in C10 cells compared with 4-3.6 cells (Table 5, row 8).

Using the optimal NGF and mAb 5C3 concentrations above, we studied TrkA PY after treatment of cells with various combinations of the ligands (Fig. 2). 4-3.6 cells were untreated (row 1) or treated with 1 nM NGF (row 2); 5 nM mAb 5C3 alone (row 3); 0.5 nM mAb MC192 alone (row 4); or a combination of both mAbs (row 5) for 15 min at 37°C. Ligand concentrations were selected from survival assays (e.g. Table 3) and pilot experiments (e.g. Table 4). Equal amounts of protein from whole cell lysates were resolved by SDS-PAGE and analyzed by western blotting.

Fig. 2A shows an anti-phosphotyrosine (anti-PY) blot. Short thick arrow indicates p140 TrkA. Notable changes in tyrosine phosphorylation of other cellular proteins can be seen induced by NGF, mAb 5C3 or 5C3 plus MC192 (thick dashed arrow); by NGF or mAb 5C3 only (short thin arrow); or by all treatments (thin dashed arrow).

Fig. 2B shows an anti-PY490 blot (DF-49 sera) recognizing specifically the Shc binding site of TrkA.

Fig. 2C shows a densitometric scanning quantification of band intensities relative to NGF treatment (average \pm standard error, n=5). Asterisk (*) indicates significant difference from untreated samples (paired student t-tests, n=5, p<0.03).

A 15 min treatment of 4-3.6 cells (TrkA⁺ p75⁺) with both 5C3 and MC192 mAbs (Fig. 2A and 2B, row 5) induced TrkA PY comparable to that induced by optimal

NGF doses (Fig. 2A and 2B, row 2). MAb 5C3 alone (Fig. 2A and 2B, row 3) caused significant changes in TrkA PY. However, mAb 5C3-induced TrkA PY is lower than that induced by NGF, or by combinations of mAbs 5C3 and MC192. Treatment with mAb MC192 alone did not cause significant changes in TrkA PY.

Other cellular proteins of sizes ranging from 40-125 kDa are also tyrosine phosphorylated in response to these ligands. Interestingly, the effect upon these unidentified substrates is ligand specific. For example NGF, mAb 5C3, or 5C3+MC192 (but not MC192 alone) cause the PY of a ~120 kDa phosphoprotein (Fig. 2A thick dashed arrow); whereas only NGF or mAb 5C3 cause the PY of a ~110 kDa phosphoprotein (Fig. 2A short thin arrow). All treatments cause the PY of a ~40 kDa phosphoprotein (Fig. 2A thin dashed arrow). With the exception of the ~40 kDa phosphoprotein, mAb MC192 alone did not cause significant and reproducible increases in PY of other proteins within the 15 minute treatment (Fig. 2A, row 4). More importantly mAb MC192 did not affect TrkA PY in a significant and reproducible manner (Fig. 2A and 2B, row 4; see statistical analysis in Fig. 2C).

Densitometry of the TrkA band of five anti-PY blots as in Fig. 2A revealed a significant increase in total PY induced by combination of mAbs 5C3 and MC192 (91% of that induced by optimal NGF) (Fig. 2C). The total PY increase induced by treatment with mAb 5C3 alone (56% of that induced by optimal NGF) is significantly higher than untreated control ($p=0.029$), and it is also significantly different than total PY increases induced by mAb combinations ($p=0.022$).

Densitometry of the TrkA band of five a -PY490 blots as in Fig. 2B (DF-49 antibody) revealed an increase after treatment with mAb 5C3 (24% of that induced by optimal NGF), which was significant compared

to untreated controls ($p=0.016$) (Fig. 2C). Treatment with mAbs 5C3+MC192 also increased PY490 (66% of that induced by optimal NGF). The PY490 increases seen after treatment with mAb 5C3, or mAbs 5C3+MC192 are significantly different from each other ($p=0.008$). Treatment with mAb MC192 alone did not cause a significant increase in TrkA PY490.

Binding of TrkA [with various concentrations of NGF (in PC12 and 4-3.6 cells); or with anti-human TrkA mAb 5C3 (in 4-3.6 cells)] leads to significant trophic signals; as assessed by cell protection in SFM, by increased receptor PY, and by reduced apoptosis and DNA degradation. The signals leading to cell survival in SFM are mediated by a K252a inhibitable tyrosine kinase activity, likely TrkA.

Concomitant binding of TrkA (with the ligands above) and of p75 (with mAb MC192) increase trophic signals synergistically, to levels equivalent to optimal NGF concentrations. When mAbs 5C3 and MC192 are combined there is a small but significant higher 4-3.6 cell survival over optimal NGF. This is likely due to the mAbs being more stable in culture at 37°C than NGF, and perhaps due to receptor/ligand recycling. The possibility of a small amount of cell division is unlikely because BrdU incorporation in response to mAb 5C3 or NGF in SFM is undetectable.

Synergy of mAb MC192 and NGF in protection from apoptosis can be explained partially by increased binding of NGF to p75 receptors (Chandler CE et al. (1984) J. Biol. Chem. 259:6882-6889). However, several arguments suggest that affinity considerations are not the sole mechanism by which p75 ligands modulate TrkA function. First, while NGF increases its affinity for p75 ~3 fold in the presence of MC192, the functional enhancement is ~200 fold (survival with 5 pM NGF +

MC192 is nearly equivalent to 1 nM NGF). Second, enhancement of p75 affinity by mAb MC192 ought to sequester NGF from TrkA and therefore a *reduction* in TrkA-mediated survival should occur rather than the observed increase. Third and most importantly, mAb MC192 enhances the biological and biochemical function of TrkA stimulated with mAb 5C3. Synergy between these mAb ligands was not due to a change in affinity or binding properties of the mAbs; because each mAb binds its receptor irrespective of and unaffected by the other (see Materials and Methods; Antibodies as NGF receptor ligands).

Functional synergy between p75 ligands and TrkA ligands (in cells expressing both receptors), together with decreased TrkA-mediated signals in TrkA⁺ p75⁺ cells compared with TrkA⁺ p75⁻ cells suggest functional interactions. Two non-exclusive mechanisms may account for the p75 effect: (i) bound p75 positively enhances TrkA signals directly or indirectly; and (ii) unbound p75 negatively modulates TrkA-mediated trophic signals directly or indirectly. Our data provides stronger support for the latter mechanism, based on the following three arguments.

First, decreased trophic signals in response to TrkA binding by mAb 5C3 were detected in 4-3.6 cells (TrkA⁺ p75⁺) when compared with C10 cells (TrkA⁺ p75⁻). Comparable data was published using fibroblasts transfected with trkA cDNA (LeSauter L et al. (1996) J Neurosci 16:1308-1316).

Second, synergistic effects occur between TrkA ligands and mAb MC192 only when the concentration of MC192 is optimized to achieve bivalent binding of all or most receptors. At low concentrations (sub-saturating) mAb MC192 does not synergize with TrkA ligands. At very high mAb MC192 concentrations poor synergy is

observed, likely because of high dose inhibition (the probability of mAb binding in a monovalent fashion). This is consistent with reports that high doses of mAb MC192 (8 mg/ml; ~40 fold higher than our optimal concentrations) can antagonize the effect of NGF on PC12 cells.

Third, protection from apoptotic death in SFM was very limited or undetectable after binding of p75 alone with NGF (in B104 cells) or with MC192 mAb (in B104; PC12 and 4-3.6 cells); and undetectable after binding with BDNF (in B104 and 4-3.6 cells). The simplest interpretation is that detectable p75 trophic signals in SFM require pre- or co-activation of TrkA. This would be consistent with a possible protein kinase that associates with p75 receptors only after TrkA activation (Canossa M et al. (1996) EMBO J. 15:3369-3376).

The mechanism by which p75 controls TrkA function probably does not involve TrkA-p75 heterodimers, because they are not likely to be induced by binding of the mAb-based ligands. However, the possibility that receptor heterodimers pre-exist on the cell membrane and are not ligand dependent can not be ruled out. Further, it is also possible that a positive modulation of bound p75 upon TrkA occurs (Canossa M et al. (1996) EMBO J. 15:3369-3376; Verdi JM et al. (1994) Neuron 12:33-745).

Previously, polyclonal anti-TrkA antisera was used to achieve ~70% of the neuronal survival afforded by optimal NGF (Clary DO et al. (1994) Mol. Biol. Cell 5:549-63). The neurons expressed TrkA and p75, but potential synergy upon p75 binding was not studied. Our results are consistent with and expand upon that data.

Although p75 has been reported to signal in the absence of TrkA binding (Carter BD et al. (1996)

Science 272:542-545; Cortazzo, MH et al. (1996) J. Neurosci 16: 3895-3899), those p75-mediated signals do not lead to trophic responses or to increased PY of TrkA as studied herein. Our results contrast with other reports wherein unbound p75 receptors did not modulate TrkA-mediated signals (Verdi JM et al. (1994) Neuron 12:33-745); and p75 binding in the absence of TrkA binding did protect from apoptosis induced by antimitotic Cortazzo, MH et al. (1996) J. Neurosci 16: 3895-3899). The different results likely are due to the presence of growth factors in these other experiments. Our results also differ to some extent from a report by Rabizadeh et al. (Rabizadeh S et al. (1993) Science 261:345-348), where p75-mediated TrkA-independent protection from apoptosis was described in NR5D (a line derived from PC12 cells) and CSM14.1 (immortalized neuronal cells), purported to lack TrkA as assessed by Northern blot analysis. However, these cells may express very low levels of TrkA which may help to explain the discrepancy.

Analysis of TrkA PY, particularly the Shc docking site PY490, confirmed that higher activity is induced upon concomitant binding of TrkA and p75. This likely is due to increased kinase kinetics, to lower tyrosine phosphatase activity, or to sustained phosphorylation of PY490. Either of these alternatives support the hypothesis of a negative modulation of TrkA enzymatic activity by unbound p75.

Based on our western blot experiments, the putative negative modulation by p75 seems to be released within a few minutes. Thus, it is unlikely that this modulation involves NF κ -b (Carter BD et al. (1996) Science 272:542-545) or JNK transcriptional pathways. Perhaps the regulation of TrkA by p75 is more direct and acts via phospholipid hydrolysis or other kinases.

Important changes in the PY of cellular proteins other than TrkA are also seen induced by ligands that afford optimal protection from apoptotic death. Some of these proteins are tyrosine phosphorylated in a
5 ligand-specific manner. The identification of these phosphoproteins may reveal differences or specificities in signal transduction induced by NGF versus antibody-based ligands; and will aid in understanding whether the putative negative modulation of TrkA is direct or
10 indirect via adapter or regulatory proteins.

There exist very few anti-receptor mAbs with agonistic activity; and even agonistic polyclonal antisera are rare. Thus, given the dimerizing ability of antibodies it seems that while receptor dimerization
15 is required, it can not alone account for agonistic function. Likely, a conformational change(s) in the structure of the receptor must also occur. We predict that mAb 5C3 affords TrkA homodimerization as well as a partial receptor conformational change(s) that lead to
20 partial agonistic signals.

Partial conformational changes are expected from the fact that mAb 5C3 likely docks onto a region of TrkA and affects the receptor differently than NGF. This is also supported by published observations that
25 mAb monovalent 5C3 Fabs function as agonists in bioassays using fibroblasts transfected with human TrkA (LeSauter L et al. (1996) J Neurosci 16:1308-1316). Further, treatment of C10 cells (TrkA⁺ p75⁻) with mAb 5C3 affords only ~80% of the trophic survival afforded
30 by treatment NGF, suggesting that mAb 5C3 and NGF are not identical TrkA ligands.

Structural analysis of mAb 5C3-TrkA and NGF-TrkA complexes may reveal the nature of the differences, and perhaps putative receptor conformational changes that
35 occur upon ligand binding.

An important and novel concept is the demonstration that functional agonism in a multireceptor system could be optimally achieved by a combination of a natural ligand and an anti-receptor antibody; or by
5 two antibodies against different constituents of the complex. This information might be useful in the design of artificial receptor agonists and antagonists, particularly for neurotrophin or other multireceptor systems.

10 The present invention will be more readily understood by referring to the following examples which are given to illustrate the invention rather than to limit its scope.

15 EXAMPLE I

Compounds which binds to NGF receptor TrkA or p75

PC12 cells were placed in 96-well plates in serum free media as described before. These neuronal-like cells undergo apoptotic death unless they are res-
20 cued by ligands of the NGF receptor TrkA or p75.

The indicated ligands were added to the wells at the concentrations shown throughout (NGF either 10 nM or 10 pM; TrkA-binding peptides C(92-96) or C(92-97) at 10 uM; control irrelevant peptide at 10 uM) (the pep-
25 tides are described in LeSauter et al., 1996, J. Biol. Chem. 271:1249); and anti-p75 receptor mAb MC192. After 72 hours cell survival was quantitated with the MTT assay.

Data was standardized to optimal protection by
30 NGF at 1 nM (100%). No treatment or control peptide treatment afforded no protection from death. Optimal NGF treatment afforded 100% survival, and lower doses of 10 pM NGF afforded only ~44% survival.

The data demonstrated that the TrkA binding pep-
35 tides protect cells from death, approaching levels of

protection afforded by 10 pM NGF; and that a combination of p75 ligands and TrkA binding peptides synergizes and affords nearly 100% protection (Fig. 3)

A representative experiment is shown, n=8, \pm standard deviation.

EXAMPLE II

Small molecules which binds to NGF receptor TrkA or p75

Cell culture

10 PC12 cells are rat pheochromocytomas expressing low levels of rat TrkA and high levels of p75 (TrkA⁺ p75⁺⁺⁺). The B104 cells are rat neuroblastomas that express p75 but do not express Trks (TrkA⁻ p75⁺⁺⁺). The 4-3.6 cells are B104 cells transfected with human trkA
15 cDNA and express equal surface levels of p75 and TrkA (TrkA⁺⁺⁺ p75⁺⁺⁺). The 6-24 cells are PC12 cells transfected with human trkA cDNA and overexpressing TrkA (TrkA⁺⁺⁺ p75⁺⁺⁺). Cell surface expression of TrkA and p75 NGF receptors was routinely controlled by FACScan
20 immunofluorescence assays.

Antibody and fragment preparation

Anti-rat p75 IgG mAb MC192 and anti-human TrkA IgG mAb 5C3 (LeSauter L et al. (1996) J Neurosci 16:1308-1316) were purified from ascites. MAb 5C3 has
25 been described as an agonist of human TrkA, and it does not bind rat TrkA receptors. These antibodies bind to their target irrespective of expression of co-receptors, and regardless of the presence of the other antibody. Purified IgGs were digested with papain
30 (Gibco, Toronto, Ontario) to yield monovalent fragments (Fabs). After inactivation of papain, Fabs were re-purified with protein G-Sepharose and KappaLock-Sepharose (Upstate Biotechnology, Lake Placid, NY). Intermediate and final products were char-
35 acterized to >98% purity. No IgG was detected in Fab

preparations. Analysis by HPLC and by size exclusion under native conditions did not reveal the presence of aggregates, even at high Fab concentrations. FACScan and other assays demonstrated that Fab preparations
5 bound their receptors in a specific and saturable fashion, indistinguishable from intact IgGs.

Cyclic NGF mimics

The NGF mimic C(92-96) is an N-acetylated (N-Ac) cyclic peptide with primary sequence YCTDEKQCY, and
10 another similar NGF mimic termed C(92-97) has primary sequence YCTDEKQACY. The C(92-96) and C(92-97) peptides are cyclic by oxidation and intrachain disulfide bonding of the Cys side chains (indicated by the underline). The C(92-96) peptide binds TrkA specifically
15 with an apparent $K_d \sim 10^{-7}$ M (LeSauter L et al. (1995) J. Biol. Chem. 270:6564). Linear peptides with the same sequences do not bind TrkA, and were prepared as controls by substituting the Cysteines with Methionines (primary sequence YMTDEKQMY). The linear control ana-
20 logs can not form disulfide bonds, do not cyclize, and are not conformationally constrained. The C(92-97)dimer is a tethered covalently linked dimer of C(92-97). Analysis by HPLC and mass spectroscopy confirmed the expected retention time and mass for a dimer.

Peptide synthesis and characterization

N-Ac peptides were synthesized by Fmoc chemistry, and purified in a preparative C18 column using reverse phase HPLC. Cyclization, quality control, and characterization of the peptides were done by HPLC and
30 mass spectroscopy (LeSauter L et al. (1995) J. Biol. Chem. 270:6564), and by nuclear magnetic resonance (NMR).

Mass spectroscopy of C(92-96) demonstrated that there were no covalent dimers or oligomers. To deter-
35 mine the aggregation state of the peptide at millimolar

concentrations *in solution*, high resolution proton NMR spectroscopy was performed at 500 Mhz (Fig. 4A). As will be reported elsewhere, we have determined the solution structure of C(92-96) to better than 0.5 Å root mean square deviation (RMSD) for backbone heavy atoms. NOESY and TOCSY spectra were consistent with a monomeric, unaggregated state. The molecular correlation time of C(92-96) was assessed. Natural ^{13}C abundance NMR relaxation parameters were measured for the alpha carbon atoms. Fitting the T_1 , T_2 and heteronuclear NOEs values using a model-free formalism produced an overall correlation time of 1.76 nanoseconds at 5°C as expected for a monomer.

A better criterion for the aggregation state of a peptide is its self-diffusion constant in solution. Translational self-diffusion of C(92-96) is dependent on the hydrodynamic properties and the size of the molecule. Isotropic diffusion measurements unequivocally identified C(92-96) as monomeric. Pulse field gradient NMR measurements of the self-diffusion coefficient (D) were determined at various peptide concentrations of 2.5, 6, or 18 mM; $T=278^\circ\text{K}$. Values of $D= 1.01 \pm 0.07$ ($10^{-6} \text{ cm}^2/\text{s}$); $D= 1.00 \pm 0.06$ ($10^{-6} \text{ cm}^2/\text{s}$); and $D= 1.04 \pm 0.05$ ($10^{-6} \text{ cm}^2/\text{s}$) were measured for 18 mM, 6 mM and 2.5 mM samples respectively (Fig. 4B).

These D values are essentially the same, indicating an identical state for the peptide. Thus, the samples remain monomeric, and peptide aggregates are undetectable in solution even at concentrations as high as 18 mM. We estimate that the self-association constant for any putative aggregate cannot be larger than 10 M^{-1} . Thus, a 10 mM solution of C(92-96) could not contain $>1 \text{ nM}$ self-aggregated dimers, if any aggregate at all.

Small molecule monovalent and bivalent partial agonists of TrkA

The genuine monovalent and monomeric TrkA ligand C(92-96) was tested for trophic support in SFM. To gauge the potency and efficacy of monomeric versus dimeric small molecule TrkA ligands a covalent dimeric analog of C(92-96) termed C(92-97)dimer was evaluated. Furthermore, to study whether TrkA receptor density influences trophic signals mediated by the NGF mimics, the ligands were assayed in parallel on cell lines that only differ in TrkA density (PC12 TrkA⁺ p75⁺⁺⁺; versus 6-24 TrkA⁺⁺⁺ p75⁺⁺⁺).

Table 6

Concomitant p75 and TrkA binding protects cells from apoptotic death

	TREATMENT	% protection	
		PC12	6-24
1	1 nM NGF	100 ± 1.8	100 ± 3.7
2	NGF 10 pM	30.4 ± 3.6	59.8 ± 3.1
3	C(92-96) 10 µM	0 ± 2.7	0.6 ± 3.1
4	C(92-97)dimer 10 µM	5.3 ± 1.6	23.1 ± 2.4
5	linear peptide 10 µM	1.3 ± 1.3	8.28 ± 1.2
6	MC192 IgG 1 nM	3.6 ± 2.5	7.38 ± 1.8
7	C(92-96) + MC192	28.0 ± 2.8	51.7 ± 5.2
8	linear peptide + MC192	5.2 ± 2.0	7.0 ± 3.8

The trophic response to 1 nM NGF was optimal in both cell types (Table 6, row 1). However, survival to 10 pM NGF was better in 6-24 cells than in PC12 cells (Table 6 row 2). This suggests that TrkA density influences ligand-induced signals, and high TrkA expression affords better potency when ligand concentrations are limiting.

The C(92-96) NGF mimic did not afford significant trophic support of PC12 or 6-24 cells; compared to control linear peptides (Table 6, rows 3 versus 5). In contrast, the C(92-97)dimer peptide afforded good

trophic support for 6-24 cells, and very low but statistically significant support for PC12 cells (Table 6, row 4).

5 MAb MC192 IgG alone afforded very low or insignificant trophic support (Table 6, row 6); but as a bivalent p75 ligand it can synergize with TrkA ligands (e.g. see Tables 2 and 3). Bivalent mAb MC192 synergized with the monomeric C(92-96) TrkA ligand in protecting 6-24 cells and PC12 cells from apoptotic death
10 in SFM (Table 6, row 7). The combination of C(92-96) plus mAb MC192 was more potent in protecting 6-24 cells. However, the efficacy of this combination was similar for both cell types, and comparable in each case to 10 pM NGF. Bivalent mAb MC192 did not synergize
15 with control linear peptides (Table 6, row 8).

Experiments using monomeric C(92-96) and C(92-97)dimer peptides in 4-3.6 cells resulted in nearly identical data to that obtained in 6-24 cells (see Fig. 5). This was expected because 4-3.6 and 6-24
20 cells express equivalent TrkA receptor densities, and indicate that the effect is not limited to a cell line. In contrast, controls using B104 cells (TrkA⁻, p75⁺⁺⁺ parental to 4-3.6) demonstrated that no protection was afforded by the peptide NGF mimics, alone or in combination with mAb MC192. These data suggest that the bio-
25 activity of the NGF mimics requires TrkA expression.

The concentration at which the peptides C(92-96) and C(92-97)dimer were effective is ~2 orders of magnitude over their apparent K_d ; at lower concentrations
30 the activity was notably reduced. However, the bioassays compare small molecule NGF mimics *versus* 1 nM NGF which is a concentration of NGF ~3 orders of magnitude over its apparent K_d . It is possible that the ligands have short half lives in solution at 37°C in bioassays

lasting 48-72 hours, or that the ligands are bound by matrix proteoglycans or carrier proteins.

Small molecule ligands and TrkA tyrosine phosphorylation

5 To further assess whether the trophic support by small cyclic peptides was indeed mediated by TrkA, tyrosine phosphorylation of the receptor was studied in 4-3.6 cells (Fig. 5). Representative anti-PY western blots are shown in Fig. 5A. A summary of densitometric
10 scanning analysis from several blots is given in Fig. 5B.

Treatment with C(92-96) peptide alone did not induce an increase in TrkA PY (Fig. 5A, lane 4) compared with control untreated cells (Fig. 5A, lane 1),
15 control linear peptide treated cells (Fig. 5A, lane 6), or bivalent mAb MC192 treated cells (Fig. 5A, lane 7). Significant TrkA PY was induced by treatment with C(92-97)dimer (Fig. 5A, lane 5). TrkA PY induced by C(92-97)dimer represented ~29% of that induced by 1 nM
20 NGF (Fig. 5A, lane 2).

Combinations of mAb MC192 and C(92-96) peptide (Fig. 5A, lane 8), or mAb MC192 and C(92-97)dimer peptide (Fig. 5A, lane 9) afforded notable increases in TrkA PY, respectively 41% and 53% of 1 nM NGF. These
25 TrkA PY signals are comparable to those induced by 10 pM NGF (Fig. 5A, lane 3). In contrast, treatment with a combination of bivalent mAb MC192 and linear peptide controls (Fig. 5A, lane 10) did not result in significant increases in TrkA PY. For statistical analysis see
30 Fig. 5B.

Monovalent partial agonists of TrkA

Monovalent TrkA ligands 5C3 Fabs and C(92-96) can be partial agonists in trophic assays. Protection from apoptosis by 5C3 Fabs was obtained with several

cell lines, suggesting that the effect is not cell line specific.

Functional responses to monomeric ligands of TrkA are evident. For example, the *genuine* monomeric small molecule C(92-96) afforded trophic support under certain conditions, although the bivalent C(92-97)dimer peptide was more efficient. Since the covalent dimeric C(92-97)dimer did not afford trophic responses at nM concentrations, it is unlikely that any C(92-96) self-aggregation in solution beyond the detection of NMR can account for the effects of this monomeric peptide.

It is also highly unlikely that the monomeric C(92-96) is not monovalent because it is too small to interact with two receptors simultaneously. It is well-established that receptor-ligand interactions generally involve contact by 4-30 side chains, and C(92-96) has 5 residues or less in the pharmacophore. Nevertheless, the intriguing possibility that the C(92-96) peptide could dimerize *after* docking remains unexplored. An answer to this question requires structural analysis of the receptor-ligand complex.

Monovalent and bivalent ligands of p75

Monovalent or bivalent p75 ligands alone do not substantially protect cells from apoptosis; and when monovalent p75 ligands are combined with TrkA ligands the trophic effect of the latter are not potentiated synergistically. However, bivalent p75 ligands do potentiate the effects of TrkA ligands (either mAb 5C3, peptidic NGF mimics, or suboptimal doses of NGF). It is possible that other p75 ligands not tested here may have different effects or synergy.

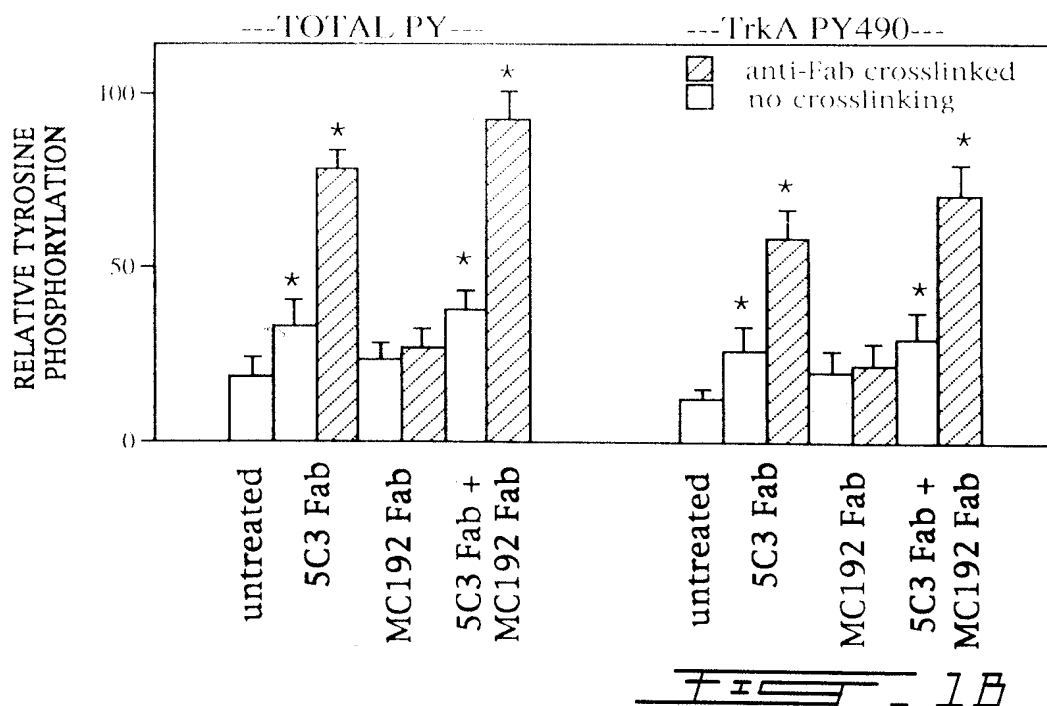
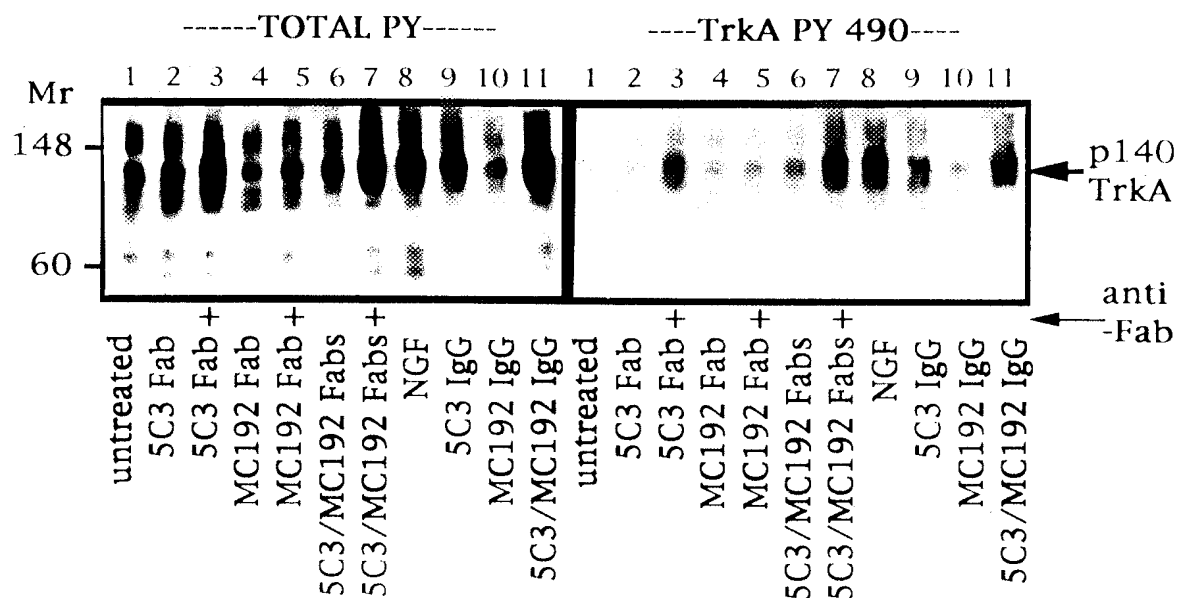
While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any varia-

tions, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

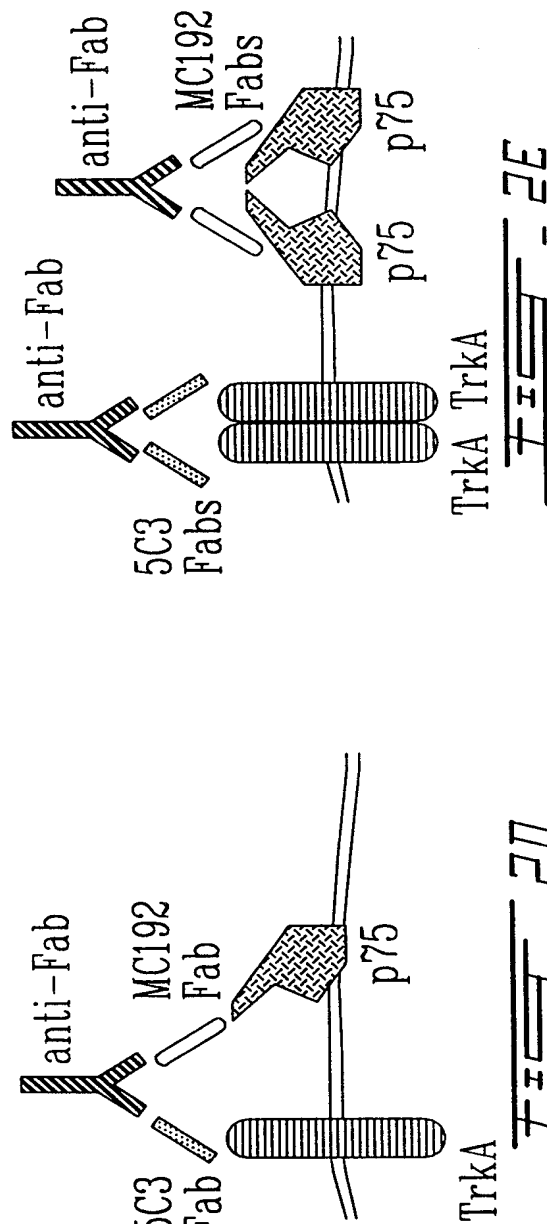
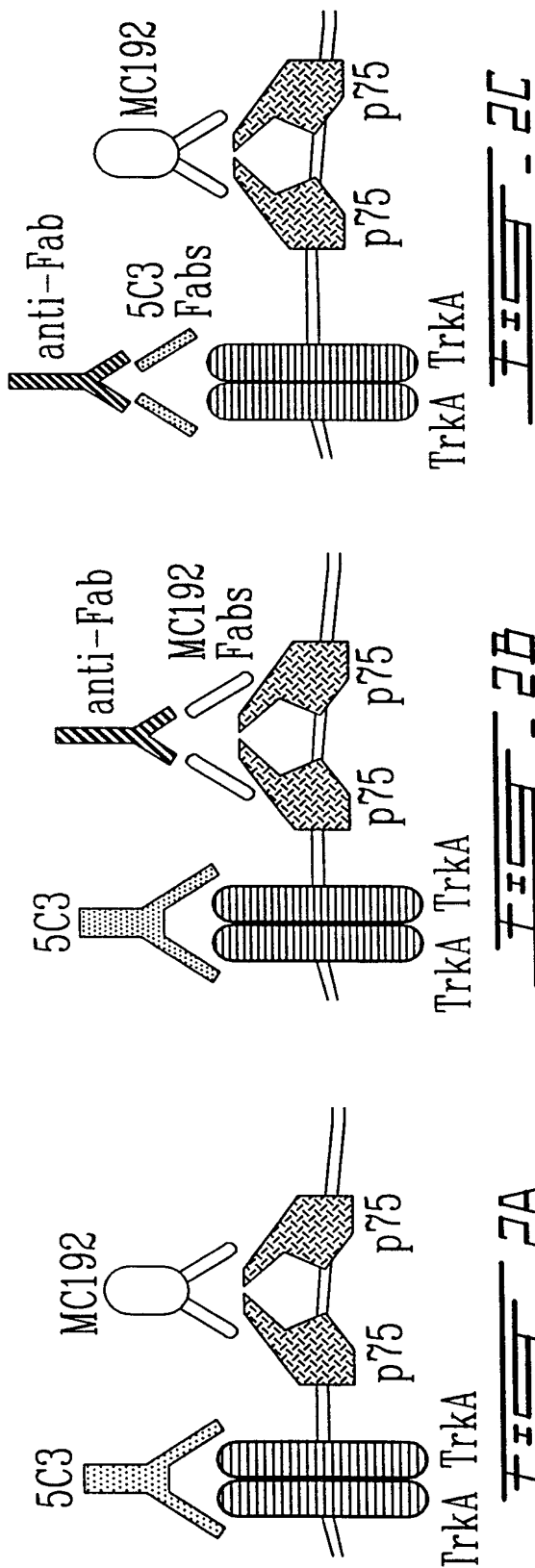
WHAT IS CLAIMED IS:

1. A pharmaceutical composition having a neurotrophic-like biological activity which comprises a first compound which binds to and/or activates at least one trk receptor, and a second component which binds to and/or activates p75 receptor, in association with a pharmaceutically acceptable carrier.
2. The pharmaceutical composition of claim 1, wherein said neurotrophic-like biological activity is that of one compound selected from the group consisting of NGF, NGF, NT-3, neurotrophins and cytokines.
3. The pharmaceutical composition of claim 2, wherein said neurotrophin is selected from the group consisting of BDNF, CNTF and GDNF.
4. The pharmaceutical composition of claim 1, wherein said trk receptor is selected from the group consisting of A, B and C.

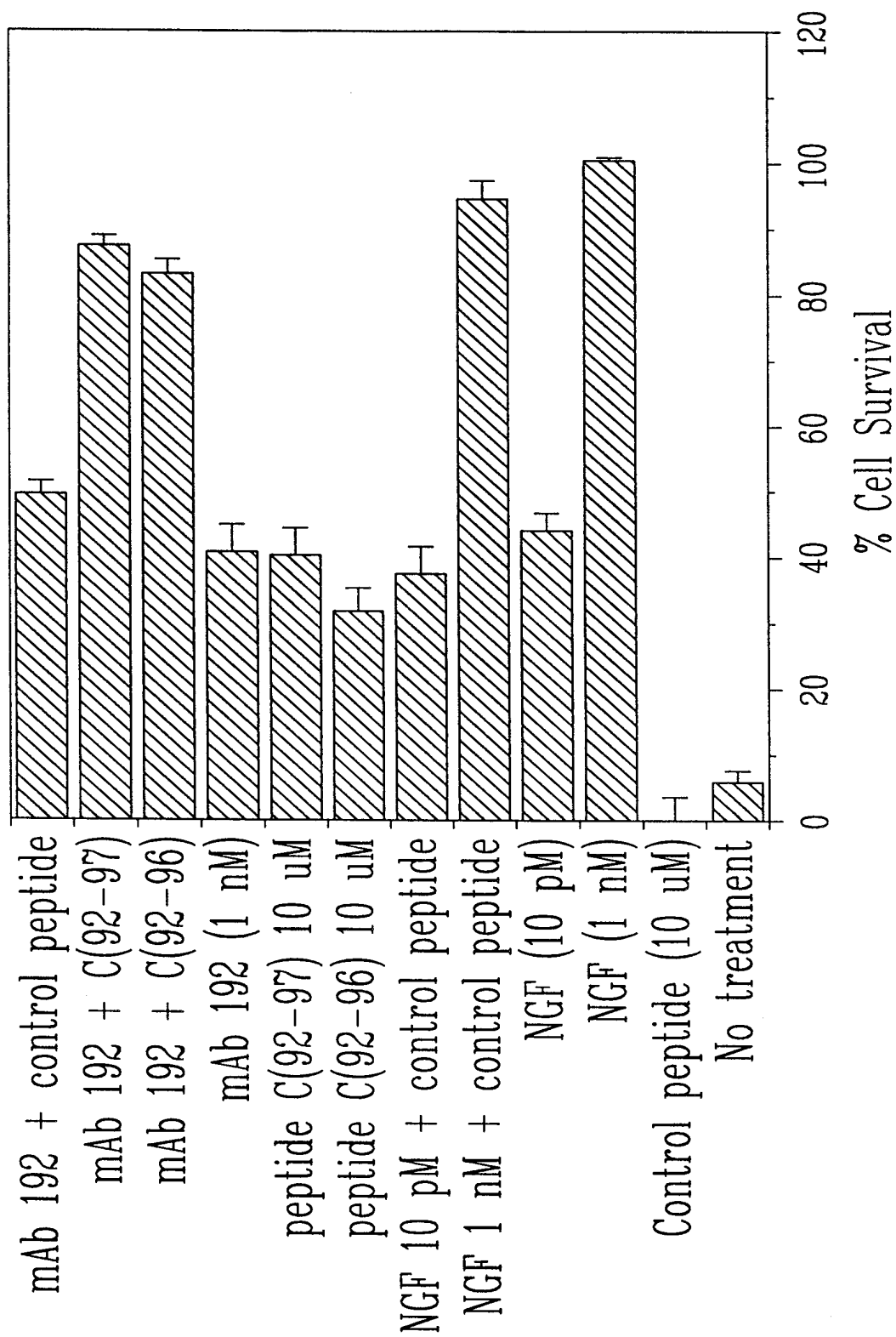
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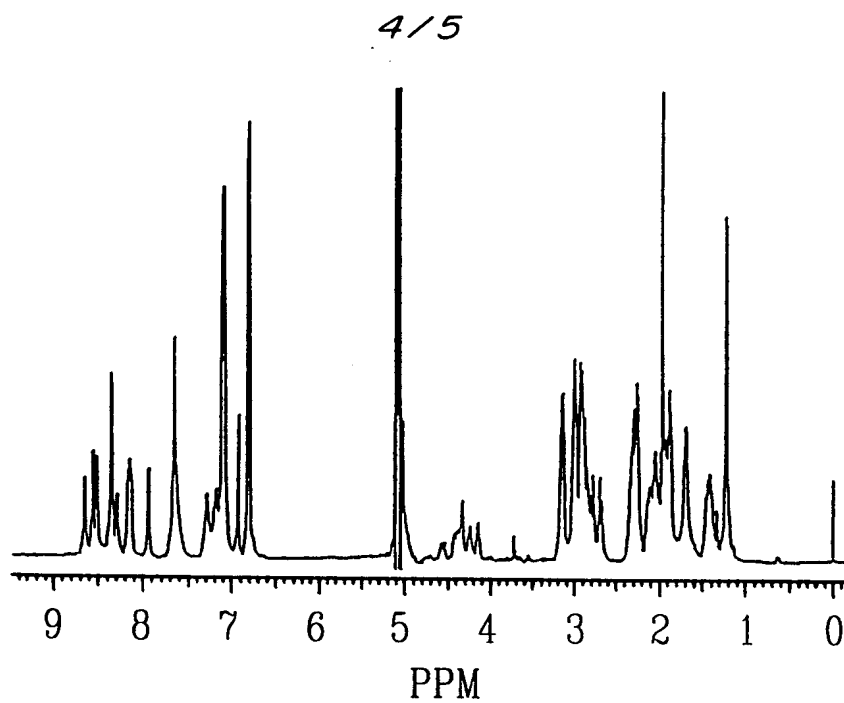


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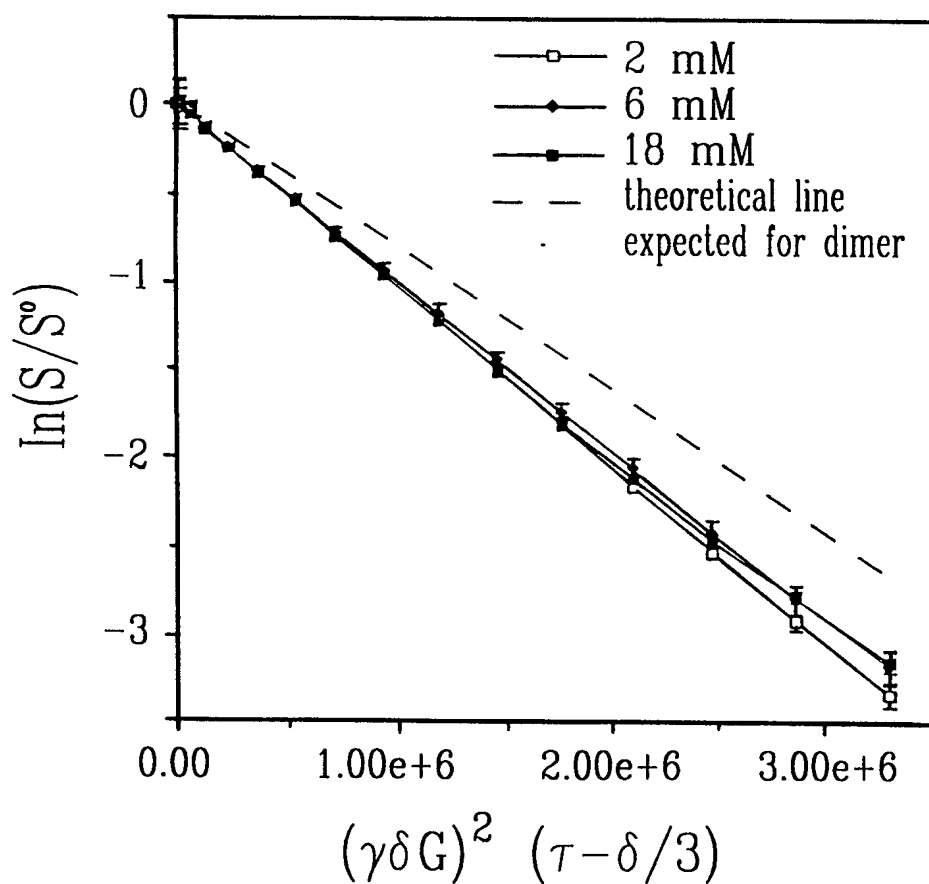


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FIG. 3

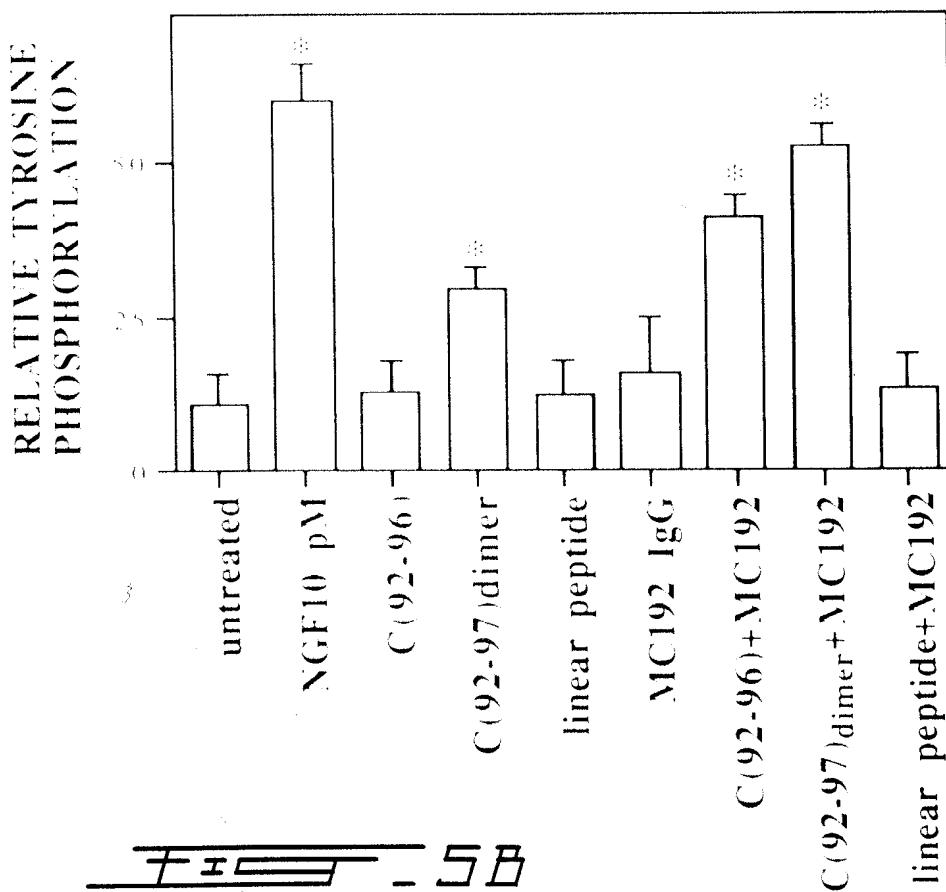
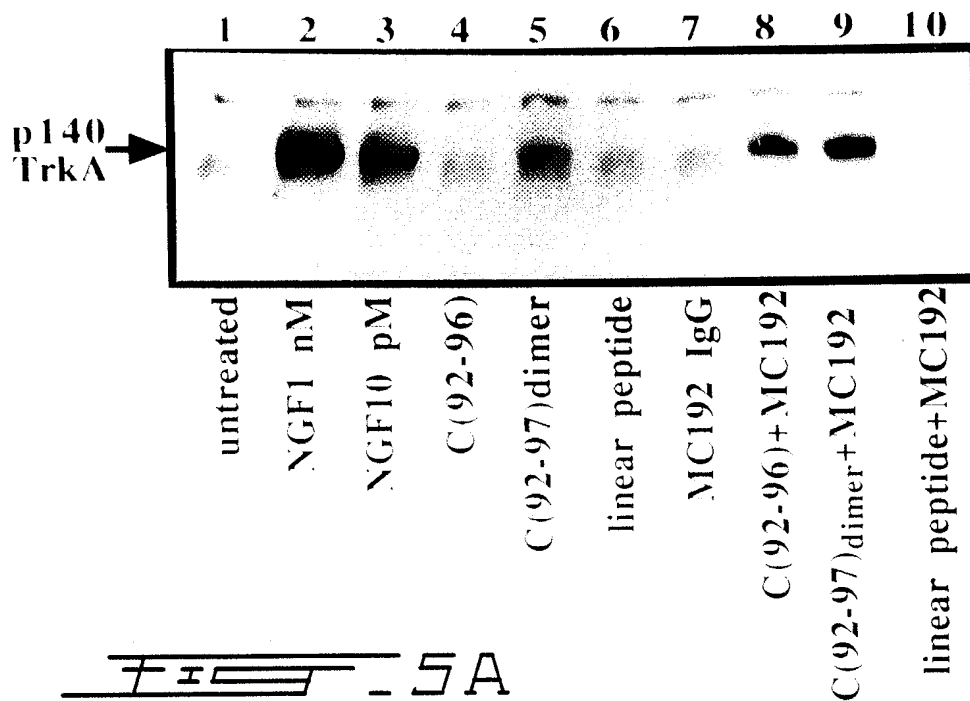


4a



4b

5/5



INTERNATIONAL SEARCH REPORT

International Application No
PCT/CA 98/00749

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K39/395 A61K38/18

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 488 099 A (PERSSON DECEASED HAKAN B ET AL) 30 January 1996 see column 7, line 3 - column 8, line 52 see column 17, line 50 - column 18, line 30; claims 1-4	1-4
X,P	MALIARTCHOUK S. ET AL: "OPTIMAL NERVE GROWTH FACTOR TROPHIC SIGNALS MEDIATED BY SYNERGY OF trKa AND P75 RECEPTOR-SPECIFIC LIGANDS" THE JOURNAL OF NEUROSCIENCE, vol. 17, no. 16, 15 August 1997, pages 6031-6037, XP002085192 see the whole document	1-4

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

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Date of mailing of the international search report

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/CA 98/00749

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>ZHENG J L ET AL: "NEUROTROPHIN-4/5, BRAIN-DERIVED NEUROTROPIC FACTOR, AND NEUROTROPHIN-3 PROMOTE SURVIVAL OF CULTURED VESTIBULAR GANGLION NEURONS AND PROTECT THEM AGAINST NEUROTOXICITY OF OTOTOXINS"</p> <p>JOURNAL OF NEUROBIOLOGY, vol. 28, no. 3, November 1995, pages 330-340, XP000674480 see figures 3,6,7 see page 333, left column, first paragraph; page 336, left column, paragraph before "discussion"</p> <p>---</p>	1-4
A	<p>WO 95 15180 A (UNIV CALIFORNIA) 8 June 1995 see page 63, line 29 - page 64, line 6; claims 1-15; figure 4</p> <p>---</p>	1-4
A	<p>WO 97 21732 A (UNIV MCGILL ;SARAGOVI H URI (CA); LESAUTEUR LYNNE (CA)) 19 June 1997 see the whole document</p> <p>---</p>	1-4
A	<p>CANOSA M. ET AL: "p75NGFR and TrkA receptors collaborate to rapidly activate p75NGFR-associated protein kinase"</p> <p>THE EMBO JOURNAL, vol. 15, no. 13, 1996, pages 3369-3376, XP002085193 see the whole document</p> <p>---</p>	1-4
A	<p>LESAUTEUR L ET AL: "POTENT HUMAN P140-TRKA AGONISTS DERIVED FROM AN ANTI-RECEPTOR MONOCLONAL ANTIBODY"</p> <p>JOURNAL OF NEUROSCIENCE, vol. 16, no. 4, 15 February 1996, pages 1308-1316, XP000645295 cited in the application see the whole document</p> <p>---</p>	1-4
A	<p>CHANDLERT C.E. ET AL: "A monoclonal antibody modulates the interaction of nerve growth factor with PC12 cells"</p> <p>THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 259, 1984, pages 6882-6889, XP002085194 cited in the application see the whole document</p> <p>-----</p>	1-4

INTERNATIONAL SEARCH REPORT

information on patent family members

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